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Direct expression of PCR products in a cell-free transcription/translation system: synthesis of antibacterial peptide cecropin

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Abstract A simple and effective methodology is proposed for direct expression of PCR-generated linear DNAs in cell-free transcription/translation systems without cloning DNA fragments in plasmids. This methodology is realized for the synthesis of the active antibacterial peptide cecropin using the synthetic coding sequence. Possible scientific applications and perspectives of the proposed approach are discussed.

Key words: Transcription; Translation; Antibacterial peptide cecropin; Polymerase chain reaction technique

1. Introduction

Translation in cell-free systems is commonly used in studies of protein biosynthesis mechanisms over a long period of time. During the last decade cell-free protein synthesis systems have been proposed for biotechnological applications [1–5]. These systems are based either on translation of isolated or in vitro presynthesized mRNA, or on combined transcription/translation where ribosomes use nascent mRNA transcribed from a plasmid or an isolated gene by endogenous (cellular) or exogenous (e.g. phage) RNA polymerases.

In most reported cases the genes for mRNA production were isolated from cells and cloned for expression. A fully cell-free format including initial chemical synthesis of a gene and its PCR amplification with subsequent direct expression in a cell-free transcription/translation system was first proposed by one of us (A.S.S.) several years ago [1]. Here we report on the practical realization of this way for the synthesis of cecropin, an antibacterial peptide of 31 amino acid residues in length. In no step did living cells participate in the process.

2. Materials and methods

2.1. Primers

All oligonucleotides were synthesized by 'Gene Assembler Plus' (Pharmacia).

Primer Pr1: 5′-CCCGCGCATGGGCTCTTGGCTGT-3′; its 5′-end contains nucleotides (underlined) complementary to the 3′-end sequence of the cecropin-coding region and the ATG triplet.

Primer Pr2: 5′-AGCCCATGCGCGGGCCGCCCT-3′; it has the 5′-end sequence complementary to the 5′-end coding region with the ATG codon (underlined) of the synthetic gene, but the stop codon is excluded.

Megaprimer (163 nt) containing regulatory elements for efficient expression of genes (the T7 promoter, the *s10* leader and Shine-Dalgarno sequences [6], as well as the additional 45 nt sequence upstream of the T7 promoter) and with its 3′-end complementary to the beginning of the cecropin gene was obtained by PCR from plasmid pET21d(+) (Novagen) carrying the synthetic cecropin gene [7]. Two primers, Pr3: 5′-CGGCCACGATGCGTCCGGCGTAGA-3′ (upstream of the T7 promoter) and Pr4: 5′-GCAGTTTTAGACAGCCAAGAGCCCATG-3′, possessing the 5′-end sequence of the cecropin-coding region with the ATG triplet at its 3′-end, were used for this purpose.

Primer Pr5 with the stop codon at the 3′-end of the cecropin gene was prepared earlier for the gene construction [7].

2.2. PCR technique

PCR for the megaprimer synthesis was carried out for 25 cycles of 1 min at 92°C, 1 min at 54°C and 0.3 min at 72°C, with 0.7 μmol each of primers Pr3 and Pr4, in 100 μl reaction volume containing 10 mM KCl, 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.2 mM each dNTP, 250 ng plasmid and 2 U Vent DNA polymerase (NEB). Multimeric cecropin genes were obtained with Pr1 and Pr2 in a similar way. A 5 μl aliquot of multimeric cecropin genes was subjected to PCR as a template with 0.5 μg of the megaprimer and 0.7 μmol of primer Pr5 for 25 cycles of 1 min at 90°C, 30 s at 54°C, 1 min at 72°C each with 2.5 U of the Taq DNA polymerase. The reaction conditions were the same as in the previous amplification procedure. The final multimeric PCR product was isolated by Wizard PCR purification resin (Promega).

2.3. Plasmid construction

The multimeric PCR product treated with the *Nco*I and *Eco*RI enzymes (BION, Moscow) was cloned in the pET21d(+) plasmid (Novagen, USA). Transformed cells were analyzed with restriction enzymes. The isolated plasmid with a dimeric cecropin gene was used in the transcription/translation system as a control experiment.

2.4. Expression in vitro

Experiments on the in vitro expression were carried out in the transcription/translation system based on the *Escherichia coli* MRE 600 S30 extract supplemented with the T7 phage RNA polymerase [8]. The reaction mixture contained 50 units RNasin, 100 units T7 polymerase, 20 μM [^{14}C]Leu (282 Ci/mol) (Amersham), 1/10 volume S30 extract, 100 μM of each amino acid and 1 mM of each NTP. A 20 μl aliquot of the PCR reaction mixture corresponding to 0.5–1 μg of the DNA synthesized was added to 50 μl reaction volume. In the control expression experiment 1 μg of plasmid with the dimeric cecropin gene was used in the same translation mixture. 10 μl aliquots were taken at consecutive time points for the estimation of [^{14}C]Leu incorporation into hot TCA insoluble material. Synthesized products were also analyzed by PAGE [9] followed by autoradiography.

2.5. Activity assay

Antibacterial activity was tested by the growth zone inhibition assay using *E. coli* strain D21 [10]. Multimeric cecropin products were cleaved with BrCN to produce active monomeric cecropin. For that purpose 200 μl of the translation mixture was clarified by centrifugation, dialyzed against 5 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.4), dried and solved in 70% CH_3COOH. After the addition of 0.5 mg BrCN the mixture was incubated overnight in the dark. Then the mixture was diluted 10-fold with distilled water, dried and dissolved in 20 μl water. 10 μl aliquots were applied to the bacterial lawn.

As a negative control, the same procedure was applied except no cecropin-encoding DNA was added to the translation system.

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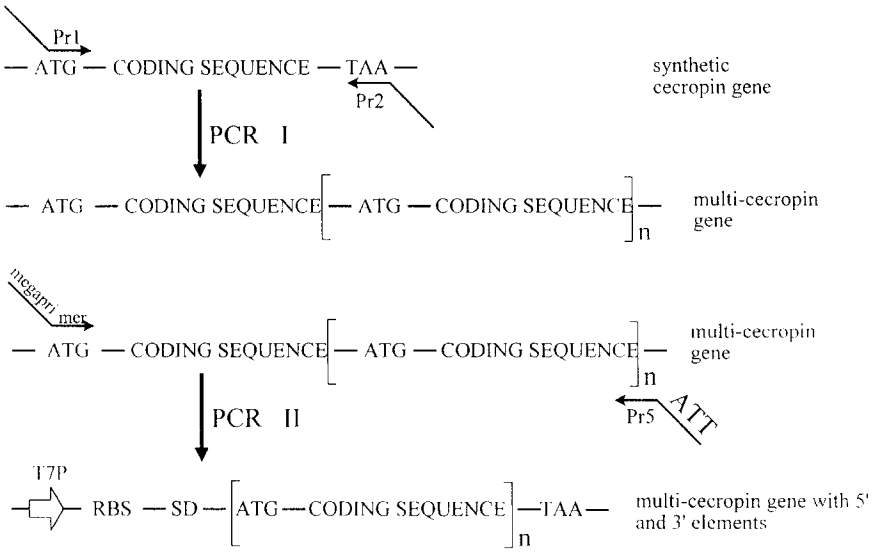

Fig. 1. General scheme of DNA construction for its cell-free expression (see Section 2).

3. Results

3.1. Production of multimeric gene construct with 5'- and 3'-elements

The following DNA sequence coding for cecropin

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          25                  50
GATCCATGGGCTCTTGGCTGTCTAAAACTGCGAAAAAACTGGAAAACTCTGCGAAAAAA
        75                  100
CGCATCTCTGAAGGCATCGCGATCGCGATCCAGGGCGGCCCGCGCTAAG
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was deduced from the amino acid sequence of cecropin (SWLSKTAKKLENSAKKRISEGIAIAIQGGPR), and the DNA fragment was synthesized earlier [7] (ATG and stop codons are underlined).

A set of multimeric cecropin genes was obtained by the modified splicing overlap extension technique [11] with the use of primers Pr1 and Pr2. A conceptually similar procedure to polymerize DNA fragments encoding decapeptides was published recently [12]. Using primer Pr2, the ATG codon for Met was inserted and the stop codon TAA excluded to produce a tandemly repeated cecropin gene without stop codons inside the linear DNA fragment (Fig. 1).

The long primer (megaprimer) containing the T7 promoter and the ribosome binding site (RBS) with the *s10* leader and Shine-Dalgarno sequences was generated by PCR from plasmid pET21d(+) [7] with the use of primers Pr3 and Pr4. The megaprimer and primer Pr5 were added to the products of the first PCR, containing up to seven repeats of the cecropin coding sequence [11], and the next PCR round resulted in production of the linear DNA with necessary elements for direct expression in a cell-free system (Fig. 1).

3.2. Direct PCR product expression

Unpurified PCR mixture (preliminarily concentrated in some experiments) was added to the *E. coli* S30 extract with the T7 RNA polymerase for direct expression of the synthesized DNA fragments in a cell-free transcription/translation system. The cecropin yield was up to ca. 3 nmol/ml (calculated for monomeric cecropin) (Fig. 2). For comparison, the DNA fragment encoding dimeric cecropin cloned in plasmid and

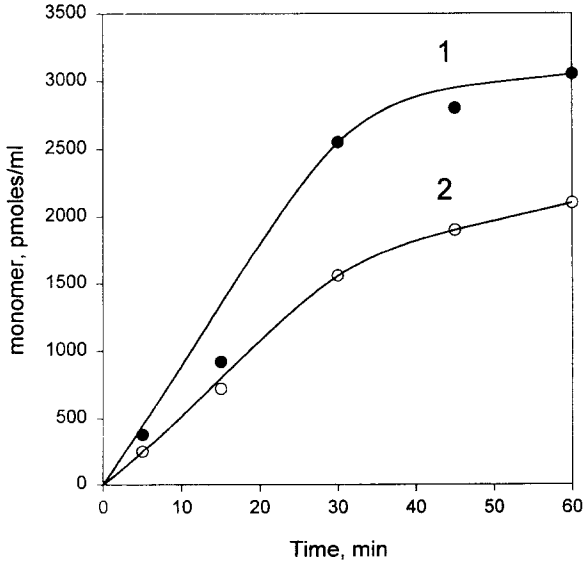

Fig. 2. Synthesis of cecropin in cell-free transcription/translation systems: (1) direct expression of PCR product (multimeric cecropin genes); (2) expression of plasmid with dimeric cecropin gene.

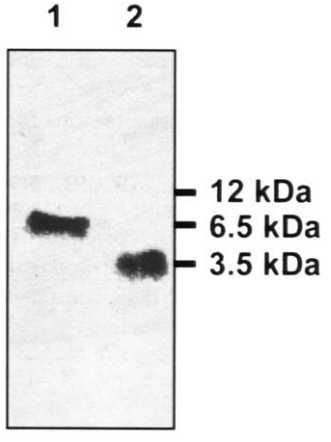

Fig. 3. Gel electrophoresis of the cell-free transcription/translation products: radioautography of the polypeptides synthesized (1) in the plasmid-directed system (dimeric cecropin), and (2) in the PCR product-directed system after CNBr cleavage (monomeric cecropin).

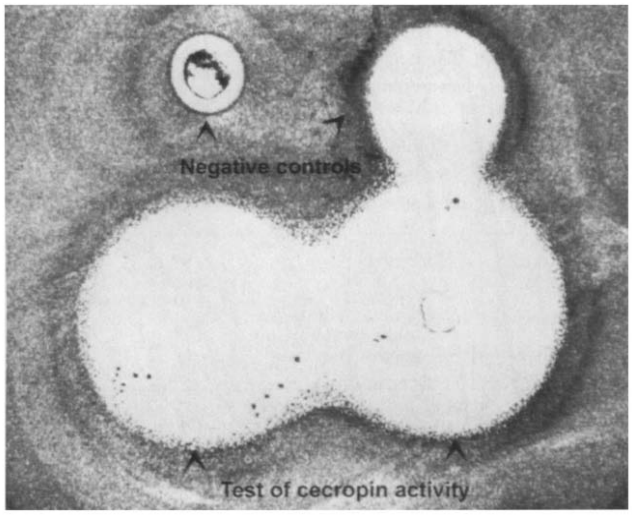

Fig. 4. Cecropin activity assay. Translation mixtures after CNBr cleavage of the polypeptide product were tested (see Section 2.5).

expressed at the same conditions gave 2.1 nmol/ml (Fig. 2). Gel electrophoresis proved the formation of a product with the expected molecular weight (Fig. 3).

3.3. Antibacterial activity of the translation product

Earlier it was shown that dimeric cecropin synthesized in the plasmid-programmed cell-free system is not active [11], at least at concentrations where monomeric cecropin displays activity. Since the active cecropin molecule does not contain methionine, the multimeric polypeptide produced in the cell-free system could be subjected to CNBr cleavage. After cleavage of the translation product, the antibacterial activity was clearly displayed in the cell growth inhibition test (Fig. 4).

4. Discussion

Earlier the DNA fragment encoding several antibacterial peptides, including cecropin, was synthesized, cloned in plasmid and expressed both in vivo and in vitro [7]. The yield of cecropin in the *E. coli* cell-free transcription/translation system from the monomeric synthetic gene cloned in plasmid was 800 pmol/ml. The expression of the DNA fragment encoding dimeric cecropin molecules cloned in plasmid gives 2100 pmol/ml of cecropin monomer. Direct PCR product expression produces up to 3050 pmol/ml of cecropin calculated per monomer. Since genetic elements at the 5′- and 3′-ends are the same in all the constructs used, obvious reasons for the best results in the case of direct expression of the PCR products can be the stability of the multimeric gene, the multimeric mRNA and/or the multimeric polypeptide product synthesized in the cell-free system. This finding correlates with the fact that cecropin fused to protein A could be successfully synthesized in vivo [7]. We believe that our PCR constructs can be further improved by addition of a stable structural element at the 3′-end of the gene in order to impede 3′-exonucleolytic degradation. Such elements can be introduced with the downstream primer in the last round of PCR.

The proposed approach can be used for direct in vitro expression of polypeptides and proteins that are unstable in living cells, or proteins that are strongly cytotoxic. By this method PCR-generated copies and/or multi-copies of genes and their fragments from genomic libraries (or genomic DNA) can be directly expressed in a transcription/translation system and then tested, without subcloning procedures. In particular, in vitro expression of DNA fragments with open reading frames for the purpose of protein identification, or test expression of some genes before construction of special vectors could be useful applications of the methodology reported here.

It is expected that the yield of polypeptides synthesized in this way can be increased several times in continuous flow cell-free (CFCF) expression systems [13] (see also [1–4,8]). A simplified version of the CFCF system, the so-called CECF (continuous exchange cell-free) system, where an exchange of substrates and products through a dialysis membrane is used instead of a continuous flow [14], may also be promising.

Multimeric DNA sequences encoding short cytotoxic polypeptides can be useful for their in vivo production following chemical or enzymatic cleavage after expression.

References

[1] Spirin, A.S. (1991) in: P. Todd, K. Sikdar and M. Bier (Eds.). Frontiers in Bioprocessing II. American Chemical Society, Washington, DC, pp. 31–43.
[2] Endo, Y., Otsuzuki, S., Ito, K. and Miura, K. (1992) J. Biotech. 25, 221–230.
[3] Kolosov, M.I., Kolosova, I.M., Alakhov, V.Y., Ovodov, S.Y. and Alakhov, Y.B. (1992) Biotech. Appl. Biochem. 16, 125–133.
[4] Volyanik, E.V., Dalley, A.I., McKay, R., Keigh, A.I., Williams, N.S. and Bustin, S.A. (1993) Anal. Biochem. 214, 289–294.
[5] Ryabova, L.A., Desplancq, D., Spirin, A.S. and Pluckthun, A. (1997) Nature Biotech. 15, 79–84.
[6] Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorf, J.W. (1990) Methods Enzymol. 185, 60–88.
[7] Martemyanov, K.A., Spirin, A.S. and Gudkov, A.T. (1996) Biotech. Lett. 18, 1357–1362.
[8] Baranov, V.I. and Spirin, A.S. (1993) Methods Enzymol. 217, 123–142.
[9] Schagger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[10] Lehrer, R.I., Rosenman, M., Harwig, S.S.S.L., Jackson, R. and Eisenhauer, P. (1991) J. Immunol. Methods 137, 167–173.
[11] Martemyanov, K.A., Yarunin, A.S. and Gudkov, A.T. (1997) Dokl. Acad. Sci. (Russia) 355 (in press).
[12] Nakajima, K. and Yaoita, Y. (1997) Nucleic Acids Res. 25, 2231–2232.
[13] Spirin, A.S., Baranov, V.I., Ryabova, L.A., Ovodov, S.Y. and Alakhov, Y.B. (1988) Science 242, 1162–1164.
[14] Davis, J., Thompson, D. and Beckler, G.S. (1996) Promega Notes 56, 14–21.